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Taurine-like Immunoreactivity in the Motor Nerve Net of the Jellyfish *Cyanea capillata*

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Abstract. Two antisera against the sulfonated amino acid taurine were applied to subumbrella tissue of the jellyfish *Cyanea capillata*. Taurine-immunoreactive nerve nets were found in both the ectoderm and endoderm. The ectoderm had two morphologically and immunocytochemically distinct populations of neurons, the motor nerve net (MNN), which was immunoreactive to the taurine-like molecule, and the diffuse nerve net (DNN), which was immunoreactive to the neuropeptide Phe-Met-Arg-Phe-NH_2 (FMRFamide). In the endoderm, immunoreactivity was found in the endodermal DNN. This localization was confirmed by double-labeling experiments, which also revealed that the endodermal DNN neurons may contain both taurine and FMRFamide-related peptide. The presence of a taurine immunoreactivity in the MNN supports the hypothesis that taurine or some chemically related compound is the neurotransmitter at synapses within the MNN of *Cyanea*.

Introduction

Cnidarians are the earliest extant animals to have a nervous system and, as such, they may provide useful information about the evolution of the nervous system and its components. Furthermore, their structural simplicity affords opportunities for studying functional aspects of these nervous systems, including the cellular mechanisms underlying chemical synaptic transmission (Anderson, 1985; Spencer *et al.*, 1989; Anderson and Spencer, 1989). A focus of considerable interest in recent years has been the identity of neurotransmitters in the Cnidaria. Neuropeptides are known to be common within the phylum (Grimmelikhuijzen *et al.*, 1989a, b; 1992), and evidence for a role of small molecules and amino acids as neurotransmitters is growing (Anctil, 1989; Scemes, 1989; Chung *et al.*, 1989; Chung and Spencer, 1990; Umbriaco *et al.*, 1990), but remains limited.

The sulfonated amino acid taurine, which is ubiquitous in animals and prokaryotes and has been implicated as an inhibitory neurotransmitter in both vertebrates (Huxtable, 1989) and invertebrates (Nistri and Constanti, 1976; Hue *et al.*, 1979; Giles and Usherwood, 1985), has recently been shown to depolarize neurons in the motor nerve net (MNN) of the scyphozoan jellyfish *Cyanea capillata* (Anderson and Trapido-Rosenthal, 1990). The mode of action of taurine on these neurons is very similar to that of the endogenous neurotransmitter, raising the possibility that taurine may serve as an excitatory neurotransmitter at these synapses. To determine whether taurine is present in the tissues of *Cyanea* and if so, to delineate its distribution, we used antisera raised against a taurine-bovine serum albumin complex (Campistron *et al.*, 1986; Madsen *et al.*, 1985). The results indicate that taurine, or a taurine-like molecule, is indeed present in the MNN and that its distribution is consistent with a role as a neurotransmitter in the *Cyanea* MNN.

Materials and Methods

Specimens of *Cyanea* were collected at the Tjärnö Marine Biological Laboratory on the west coast of Sweden. Pieces of perirhopalial tissue (Anderson and Schwab, 1981) were removed from the animal and pinned out to prevent curling. In some preparations the myoepithelium that envelops the MNN neurons was removed to expose the nerve net (Anderson and Schwab, 1984). Tissues were

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fixed for 3 h in freshly prepared 5% glutaraldehyde in 0.05 *M* Na-cacodylate buffer containing 1% sodium metabisulphite and 2.4% sodium chloride (pH 7.5). After fixation, the tissues were given three 15-min washes in Tris-buffered saline (TBS; 0.05 *M* TRIS-HCl buffer, pH 7.5, 1% sodium metabisulphite and 2.4% sodium chloride), followed by 30 min in 0.1 *M* sodium borohydride in TBS, then a further three 15-min rinses in TBS. Twelve specimens from 5 to 20 cm in diameter were used for immunocytochemical investigations.

Samples were incubated for 4–6 days in rabbit anti-taurine antisera diluted 1:200 (Chemicon) or 1:1000 (Immunotech S.A.) in TBS with 0.2% Triton X-100 (TBS/TX) and 1% bovine serum albumin (BSA). After three 15-min rinses in TBS/TX, the samples were incubated for 3 h with fluorescine isothiocyanate- (FITC)-conjugated swine anti-rabbit IgG (Dakopatts, Denmark) diluted 1:10 in TBS. The samples were then given three 15-min rinses in TBS, stained in a 1% solution of Evans blue (Merck) in phosphate-buffered saline (PBS), pH 7.4, rinsed for 2 h in PBS, and mounted in phosphate-buffered glycerol. Specificity was tested by preabsorbtion of antiserum with 1 μM taurine-glutaraldehyde-BSA conjugate.

Double labeling with rabbit antisera raised against taurine and the neuropeptide FMRFamide was carried out in the manner developed by Würden and Homberg (1993). Specifically, tissues were first stained with antibodies to taurine, and the location of the primary antibody was visualized by a 3-h incubation with Texas-red-conjugated donkey anti-rabbit IgG (Jackson Immuno Research) diluted 1:40. After a 3-h incubation in rabbit IgG (Dakopatts) diluted at 1:25, the tissues were then incubated for 24 h with biotinylated (Bayer and Wilcheck, 1980) anti-FMRFamide antibodies (Incstar), diluted 1:800. The FMRFamide immunostaining was then visualized by treatment with streptavidin-FITC (Dakopatts) at 1:20 for 3 h. All light microscopical observations were made with a Leitz Aristoplan microscope.

The specificity of taurine antiserum from Chemicon has been characterized by the company. Cross-reactivity with glutaraldehyde-conjugated hypotaurine was 0.067 (1:15) and was less than 0.002 for other glutaraldehyde-conjugated amino acids including GABA, beta-alanine, aspartate, glycine, cysteine, and glutamate.

Results

Light microscopy

Taurine-like immunoreactivity (Tau-IR) was found in both the ectoderm and endoderm of the perirhopalial tissue of *Cyanea*.

Ectodermal-specific Tau-IR was found in neurons and, to a lesser extent, in myoepithelial cells. Ectodermal myoepithelial cells in this species contain a large central vacuole (Anderson and Schwab, 1981). Immunoreactivity was restricted to the narrow layer of cytoplasm that surrounds each vacuole; vacuolar contents were not immunoreactive. The Tau-IR neurons were large, bipolar cells with lengths up to 2 mm, cell-body diameters of 15 to 20 μm, and axonal diameters from 1 to 5 μm (Fig. 1A). These were clearly motor nerve net (MNN) neurons (Anderson and Schwab, 1981) and were easily distinguished from the FMRFamide-immunoreactive (FMRF-IR) cells that form the diffuse nerve net (DNN) (Fig. 1C), the other nerve net present in the perirhopalial tissue ectoderm. In the MNN, synapses occur wherever two neurons are in physical contact with one another (Anderson, 1985), and, as can be seen in these micrographs (Fig. 1B), such contacts are abundant. The Tau-IR within the MNN was restricted to the perirhopalial tissue; although MNN neurons are known to extend into the radial and circular muscle bands that surround the perirhopalial tissue (Anderson and Schwab, 1981), no Tau-IR neurons were found in the radial or circular muscle bands.

In the endoderm, at least two cells types were immunoreactive. One was a population of bipolar neurons. These cells, which had cell-body diameters of 10 to 15 μm and axon diameters of 0.5 to 2 μm, were at least 0.6 mm long and formed a loose nerve net (Fig. 1D). Their overall appearance is consistent with that of the diffuse nerve net (DNN) known to be present in this tissue. The other obviously immunoreactive endodermal cell type was more difficult to characterize. The cells in question occurred relatively densely, and their immunoreactivity appeared as a rather amorphous, frequently circular mass. Whether this mass represents an intracellular compartment of the cell or the true dimensions of the cell was not clear. Both of these cell types were surrounded by a low level of background immunoreactivity interspersed with occasional nonfluorescent areas that are presumably spaces in the endodermal epithelium.

Preabsorbtion of antiserum with taurine-GA-BSA conjugate completely abolished all immunostaining.

Double labeling

Double labeling revealed two distinct nerve nets in the ectoderm. Again, Tau-IR was restricted to MNN neurons, whereas FMRF-IR was localized to a separate population of smaller, multipolar cells (Fig. 2A). FMRF-IR was also evident in the marginal rhopalia and in regions covered by the circular and radial muscle bands. At no time were the two signals co-localized in the ectoderm.

In the endoderm, both antibodies stained what appeared to be the DNN. In smaller animals, the neurons were FMRF-IR, but in larger animals they were apparently Tau-IR. In one specimen, both FMRF-IR and Tau-IR were evident in the same cells, indicating co-localization (Fig. 2B).

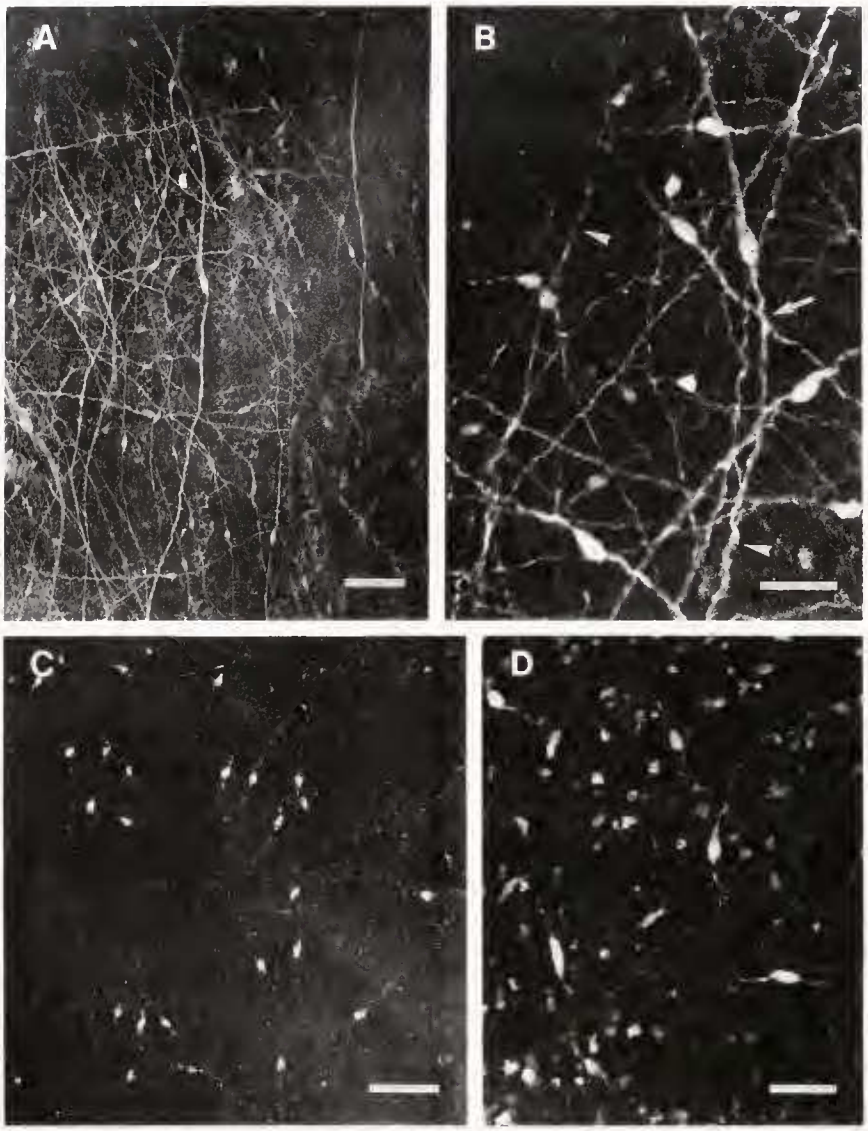

Figure 1. Whole-mount immunostaining of perirhopalial tissue of *Cyanea capillata* (A) Low power micrograph of Tau-IR in the ectoderm. Neurons in the MNN stain readily. Scale bar = 0.1 mm. (B) Micrograph of the Tau-IR MNN in the ectoderm. Several apparent contact sites between the axons (arrows) and thinner elements twined together (arrowheads) can be seen. Scale bar = 50 μm. (C) Low-power micrograph of FMRFamide-immunoreactive diffuse nerve net (DNN) in the ectoderm. Scale bar = 0.1 mm. (D) Tau-IR in the endoderm of the perirhopalial tissue. Immunoreactivity was present in bipolar neurons and in undifferentiated endodermal cells. Scale bar = 50 μm.

Discussion

In cnidarians, nerve nets are located under an overlying epithelium that forms a permeability barrier for pharmacological agents and microelectrodes. The ectodermal MNN in the perirhopalial tissue of *Cyanea* is one of the very few instances in which a coelenterate nerve net can be exposed, permitting access for electrophysiological and pharmacological studies (Anderson and Schwab, 1984; Anderson, 1985). The size of the neurons makes them suitable for electrophysiological recordings. The accessibility of this nerve net and, in particular, its synapses provides a useful preparation for studying the pharmacology of chemical neurotransmission in a cnidarian.

The MNN is a plexus of large bipolar neurons that innervates the swimming muscle bands and serves as the pathway to coordinate swimming motor activity. Synapses between the MNN neurons are fast, chemical synapses that are bidirectional (Anderson, 1985). Previous work (Anderson and Trapido-Rosenthal, 1990) has implicated taurine or a closely related molecule as a potential neuro-

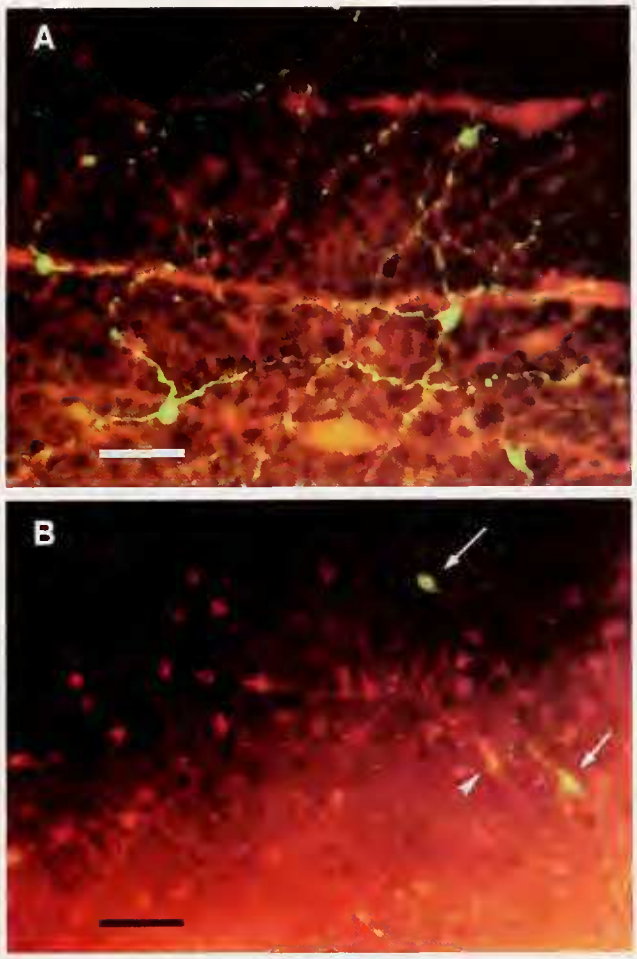

Figure 2. Double labeling. (A) Whole mount of perirhopalial tissue ectoderm, stained with antibodies against taurine (red) and FMRFamide (green). The clear anatomical separation between the Tau-IR MNN neurons and FMRFamide DNN neurons is evident. (B) Endodermal tissues stained in the same manner. The majority of cells in this preparation are Tau-IR. Two neurons (arrows) were more (yellow) or less (green) intensely immunoreactive to FMRFamide, but at least one (arrowhead) was reactive to both antibodies. Scale bars = 50 μm.

transmitter at these synapses. The current investigation provides immunocytochemical evidence that a taurine-like molecule is indeed present in MNN neurons and in an endodermal nerve net, but is not present, at least in detectable quantities, in neurons of another ectodermal nerve net, the diffuse nerve net (DNN). This was particularly obvious in the double-labeling experiments, in which the ectodermal FMRFamide-IR was clearly restricted to the DNN (Anderson *et al.*, 1992). Although Tau-IR was also present in the ectodermal myoepithelial cells, its absence in the DNN indicates that taurine is not a constituent component of all nerve nets in this animal. This distinction is important because taurine acts as an osmoregulator in many marine organisms (Thurston *et al.*, 1980). If it were serving the same function in *Cyanea*, one might expect it to be widespread in different cell types and present in all nerve nets.

The presence of Tau-IR in the endodermal DNN in *Cyanea* was unexpected considering that immunoreactivity to antibodies raised against the sea anemone neuropeptide AnthoRFamide was found in these neurons (Anderson *et al.*, 1992). In the present study, endodermal Tau-IR neurons also were found to be immunoreactive to antibodies to FMRFamide. In addition, however, morphologically similar neurons in larger animals were found to have Tau-IR, and one specimen showed apparent co-localization of the two transmitter candidates. It may be worth further investigations to find out if there is a progression from an FMRFamide or AnthoRFamide-like peptide to taurine (or a related compound) as the animal grows. To meet the requirement for faster transmission in a large medusa, a switch from peptidergic metabotrophic receptors to fast excitatory ionotrophic receptors would be functional. In either case, it is clear from this work that cnidarian synapses may have a hitherto unappreciated complexity in the number of neurotransmitters present in single neurons.

Tau-IR was also found in a very abundant, non-neuronal cell type in the endoderm (Fig. 1D). The identity of this cell is unclear. The presence of Tau-IR in these cells, and perhaps all endodermal cells if the light background fluorescence is indeed indicative of low levels of taurine, may imply that it has an alternative function such as osmoregulation. It is also possible, however, that some of the small circular profiles represent interstitial cells differentiating into DNN neurons.

The antibody used in the light microscopical component of this study is known to have low cross reactivity to the most abundant metabolites of taurine including hypotaurine and cysteine. However, one cannot as yet exclude the possibility that the antigen is a closely related compound or a small taurine-containing oligopeptide (Marnela *et al.*, 1985). Free taurine is, however, an abundant constituent of MNN neurons (Anderson and Trapido-Rosenthal, unpub.).

The MNN extends over the entire subumbrella surface, forming a network that connects all eight marginal ganglia, or rhopalia, with the circular and radial swimming muscle bands. To do this, the nerve net must transit the muscle bands, and individual, Lucifer-yellow-filled neurons have been seen to extend from the perirhopalial tissue into radial muscle bands (Anderson and Schwab, 1981). However, Tau-IR neurons were never observed in either the radial or circular muscle bands. Although the MNN neurons located within the confines of these muscle bands may employ a different neurotransmitter in this region, it is also possible that failure to observe Tau-IR in these areas is due to a technical problem. To get adequate stain-

ing of neurons in these preparations, they had to be incubated with the antibodies for as long as 6 days. In contrast, anti-FMRFamide antibodies usually penetrate the tissues easily, typically requiring 24 h (Anderson *et al.*, 1992). The staining difficulty may reflect either a low antibody titer or poor penetration by anti-Tau antibody. In either case, the thick layer of muscle that overlies the MNN in the radial and circular muscle bands may have compromised the ability of the anti-Tau antibodies to reach their targets in this region.

The major conclusion of this study is that MNN neurons are Tau-IR. This, together with electrophysiological evidence that taurine depolarizes the MNN neurons in a manner consistent with that of the endogenous neurotransmitter, provides compelling evidence that taurine, or a taurine-like molecule, is the neurotransmitter in *Cyanea*. This possibility has evolutionary implications. Taurine is one of the most abundant amino acids in the animal cell, and it is conceivable that carnivores and scavengers developed olfactory receptors for taurine very early in evolution. Receptors for taurine are, indeed, known to be present on the olfactory antennae of lobsters (Derby and Atema, 1982), and one could envisage how neurotransmitter receptors might have developed from external chemoreceptors (Carr, 1989).

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